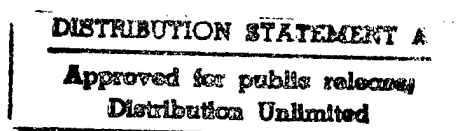


1997

FINAL TECHNICAL REPORT
to
OFFICE OF NAVAL RESEARCH

Grant: ONR N00014-96-1-0666
PI. Robert A. Andersen
Title: Marine Phytoplankton Culture Collection: Resources for Algal Biotechnology

The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) has provided ONR-funded researchers with starter cultures as well as frozen cell pellets from large volume batch cultures. The ONR-funded scientists were conducting research in the areas of biotechnology and nanofabrication. These scientists include: Dr. Anna Nadolska-Orczyk and Dr. David Rhodes at Purdue University; Dr. Douglas Gage and Mr. Wayne Hicks at Michigan State University; Dr. Andrew Hansen at the University of Florida; Dr. Thomas Leustek at Rutgers University; Dr. "Woody" Hastings at Harvard University; Dr. Emma Gonzalez at University of California-Los Angeles. In addition, we provided these and other ONR-supported research scientists with information and advice regarding the culture of marine phytoplankton. The information and advice enabled these scientists to grow phytoplankton more successfully and thereby improve research productivity. We also provided direct training for Mr. Wayne Hicks, Ph.D. student at Michigan State University, when he attended our intensive one-week course entitled "Marine Phytoplankton Culturing Techniques." This course included detailed information on the isolation, purification, growth and maintenance of algal cultures. Briefly, Mr. Hicks gained knowledge and experience in handling algal cultures with aseptic techniques, treating cells with antibiotics to obtain axenic strains, lectures on trace metals and chelators, discussions on the importance of regulating carbon dioxide, and growing cells from small scale (test tubes) to large scale (1-100 liters). Because the class was small (8 students) and because there were two instructors (Dr. Robert R.L. Guillard, Dr. Robert A. Andersen), Mr. Hicks received considerable individual attention not only on the scheduled topics but also with respect to the organisms that he was working directly with. We also isolated some new strains of marine phytoplankton that may be important for biotechnology and nanofabrication. These include, among many, an apparently new coccolithophorid species belonging to the genus *Ochrosphaera*. Finally, this ONR support provided funds to support the general operations of the CCMP. Our "List of Strains" (attached) was published as a supplement to the Journal of Phycology in December, 1997, and this grant helped support this publication.



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FINAL REPORT OFFICE OF NAVAL RESEARCH

MOLECULAR STUDIES ON MARINE ALGAE

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TITLE: MOLECULAR STUDIES ON MARINE ALGAE

LONG-TERM GOALS

One long term goal was to provide an evolutionary framework for chromophyte algae that was based upon sound scientific evidence and that could be used with confidence in addressing problems specific to chromophyte algae. Knowledge of relationships among organisms is powerful because the more closely related two organisms are, the more similar will be their genes and their expressed traits. Research, especially applied research such as biotechnology and nanofabrication, often requires examination of closely related organisms which may have more desirable expression of a trait than that of the initially discovered organism. Variants exist, however, and this may be due to "normal" genetic variation, mutations, lack of expression of genes, the lack of genes or the multiple independent "invention" of genes. Examination of ever-present "house-keeping genes (e.g., 18S rRNA gene) provides an independent means for demonstrating evolutionary relationships when target genes (e.g. bioluminescence, coccolith formation) may be unknown or absent. The resulting relationships, when compared to the genes or traits of interest, may provide insights that explain the variants observed.

Molecular techniques provided new and independent means for testing hypotheses concerning the evolutionary relationships of chromophyte algae and for proposing new evolutionary hypotheses. Species living today have common ancestors, and the occurrence of specific traits (e.g., bioluminescence, coccolith formation) (1) may be traced to a single common ancestor and thereby implying a single evolutionary "invention" or (2) the occurrence of specific traits may not be traced to a single common ancestor and thereby implying multiple independent evolutionary "inventions."

SCIENTIFIC OBJECTIVE

One goal of this project was to provide an evolutionary framework for bioluminescent and nonbioluminescent dinoflagellates using an independent molecular data base, thereby providing information regarding the origin(s) of bioluminescence amongst dinoflagellates. A second goal of this project is to provide an evolutionary framework for coccolith-bearing and noncoccolith-bearing haptophytes (=prymnesiophytes) using an independent molecular database, thereby providing information regarding the origin(s) of coccolithophorids. To meet these objectives, other chromophyte algae were examined to provide a sound evolutionary framework.

APPROACH

A wide range of dinoflagellate and haptophyte algae were being selected from those species which were in culture or found alive in field samples. For species in culture, DNA was extracted from a cell pellet, purified using a "Gene Clean" kit, and the 18S rRNA gene was amplified using PCR protocols and terminal gene primers. For field samples, individual cells were isolated by micropipette, the cells were disrupted, and the 18S rRNA gene was amplified using PCR protocols and terminal gene primers. The primary PCR product was purified by gel electrophoresis, and secondary amplifications were made using PCR protocols and selected internal and terminal sequencing primers. The sequencing primers were biotinylated and the secondary amplifications were randomly terminated using dideoxynucleotides. The reaction products were separated by gel electrophoresis and transferred to a nylon membrane. The nucleotide fragments were visualized using chemiluminescence by treating the biotinylated primers with streptavidin, biotinylated alkaline phosphatase and lumigen, and by exposing an X-ray film to the membrane. The gene sequence for the reaction was then determined, and the sum of all reactions provided the full nucleotide sequence in both directions (5' → 3' and 3' → 5'). Gene sequences were entered into a computer database, the sequences of several organisms were aligned, and a phylogeny was constructed using PAUP (Phylogenetic Analysis Using Parsimony). Support for the phylogenetic tree branches was determined by conducting a bootstrap analysis.

SCIENTIFIC RESULTS

The 18S rRNA gene sequences for 19 dinoflagellate sequences and 8 haptophyte sequences were obtained; some dinoflagellate sequences were obtained in collaboration with Gary Saunders and David Hill (University of Melbourne) and some haptophyte sequences were obtained from Linda Medlin (Alfred Wegener Institute). Analysis of the dinoflagellate data is shown in Figure 1. The bioluminescent *Noctiluca scintillans* diverges most deeply of all dinoflagellates in the tree. All other dinoflagellates fall into about four, often weakly supported, groups. One group contains most of the bioluminescent species which were examined (*Alexandrium*, *Pyrocystis*, *Ceratium*, *Ceratocorys*, and *Gonyaulax*); the nonbioluminescent *Amphidinium* and *Cryptothecodinium* appear to belong to this group. The bioluminescent *Polykrikos* is strongly grouped (100%

bootstrap) with the nonbioluminescent *Gymnodinium mikimotoi*. The other species of *Gymnodinium* form a separate group with *Lepidodinium* and this entire group is weakly associated with the symbiotic dinoflagellates belonging to the genus *Symbiodinium*. Thus, bioluminescence appears to be an ancient trait as evidenced by its appearance in the "primitive" *Noctiluca*. One interpretation is that bioluminescence, as a trait, has been lost several times during the evolutionary history of dinoflagellates. An alternative hypothesis is that bioluminescence has been "invented" independently at least three times during the evolutionary history of dinoflagellates. This second hypothesis may be less favorable because it requires multiple "inventions" - constructive (inventing) evolution is generally considered to be more difficult than reductive (loss of character) evolution.

The data also suggest that (1) the genus *Gymnodinium* requires taxonomic revision and (2) thecate and nonthecate dinoflagellates are polyphyletic.

Analysis of the haptophyte data base suggests that coccolithophorids are not a monophyletic group because *Emiliana* is more closely related to *Phaeocystis* than it is to *Coccolithus*, *Pleurochrysis* and *Crucioplacolithus* (Fig. 2).. One explanation is that *Phaeocystis* evolved from a coccolithophorid ancestor by the loss of coccolith production and the gain of colony formation. This may suggest that coccolith formation evolved only once, but the current data are too weak to resolve this possibility. If coccolith formation evolved only once, it seems likely that an early evolutionary radiation led to two distinct lineages of coccolith-bearing algae. Taxa that are probably crucial to evaluating this hypothesis were not available for study. Some confirmation of these results are seen in an unpublished data set of Fugiwara et al. where the *rbcL* gene sequence was determined for approximately 30 taxa. The *rbcL* data also show a polyphyletic nature for coccolithophorids, with *Isochrysis galbana* showing a close relationship to *Emiliana huxleyii*. Unfortunately, we did not examine *Isochrysis* and they did not examine *Phaeocystis*.

The haptophyte data also show the presence of an unidentified coccoid alga that is very closely related to three *Pavlova* spp., and this is the first report of a nonflagellate member of the *Pavloales* group.

ANCILLARY STUDIES

The equipment purchased with this grant have been used in other research projects and to modernize the Provasoli-Guillard National Center for Culture of Marine Phytoplankton. The equipment was used in conjunction with two NSF research grants and led to: (1) the discovery of a new algal class, the Pelagophyceae (see Andersen et al. 1993), (2) the discovery of a reduced flagellar lineage amongst the Chromophyte algae (see Saunders et al. 1995), (3) molecular documentation of extensive biodiversity amongst marine picoplankton (see Potter et al. 1996), (4) a re-evaluation of the class Eustigmatophyceae with the description of two new species (see Karlson et al. 1996, Brett et al. in prep.), (5) a molecular examination of the classes Raphidophyceae and Xanthophyceae (see Potter et al. in prep.), (6) the description of two new species of

Ochromonas (see Andersen et al. in prep.), (7) an examination of intraspecific variation in *Pelagomonas calceolata* using strains from all the major oceans (in prep.), etc. The modernization of the CCMP has allowed the collection to provide DNA to customers, a feature that is now advertised on the World Wide Web. Furthermore, we are beginning to use DNA fingerprinting and gene sequence data when characterizing strains.

FIGURES AND LEGENDS

Figure 1. Phylogenetic tree showing the evolutionary relationships of dinoflagellates based upon comparisons of the nucleotide sequences of their 18S rRNA gene. Sequences determined as part of this study = *. Bioluminescent organisms = B. Numbers above branches represent bootstrap values (100 replicates); branches with bootstrap values less than 50% have been collapsed. The tree is rooted with four apicomplexan taxa (*Theileria annulata*, *Sarcocystis muris*, *Perkinsus marinus*, *Perkinsus* sp.). Tree length = 1678, consistency index = 0.548, retention index = 0.572.

Figure 2. Phylogenetic tree showing the evolutionary relationships of haptophytes (=prymnesiophytes) based upon comparisons of the nucleotide sequences of their 18S rRNA gene. Sequences determined as part of this study = *. Coccolithophorids = C. Numbers above branches represent bootstrap values (100 replicates); branches with bootstrap values less than 50% have been collapsed. The tree is rooted with *Chlamydomonas reinhardtii*. Tree length = 679, consistency index = 0.722, retention index = 0.741.

PUBLICATIONS

Refereed Journal Publications

Andersen, R.A., Saunders, G.W., Paskind, M.P., and Sexton, J.P. 1993. The ultrastructure and 18S rRNA gene sequence for *Pelagomonas calceolata* gen. et sp. nov., and the description of a new algal class, the Pelagophyceae classis nov. *J. Phycol.* 29: 701-716.

Saunders, G.W., Potter, D., Paskind, M.P. and Andersen, R.A. 1995. Cladistic analyses of combined traditional and molecular data sets reveal an algal lineage. *Proc. Nat. Acad. Sci. U.S.A.* 92: 244-248.

Refereed Journal Publications in Press

Potter, D., LaJeunesse, T.C., Saunders, G.W. and Andersen, R.A. 1996. Convergent evolution masks extensive biodiversity among marine coccoid picoplankton. *Biodiversity and Conservation* 5: (in press).

Karlson, B., Potter, D., Kuylensstierna, M. and Andersen, R.A. 1996. Ultrastructure, pigment composition and 18S rRNA gene sequence for *Nannochloropsis granulata* sp. nov. (Monodopsidaceae, Eustigmatophyceae), a marine

ultraplankton isolated from the Skagerrak, North East Atlantic Ocean. *Phycologia* (in press).

Papers in Preparation for Referred Journals

- Andersen, R.A., LaJuenesse, T.C., and Potter, D. Ultrastructural observations and 18S rRNA gene sequences for two new marine species, *Ochromonas provasoli* sp. nov. and *Ochromonas moestrupii* sp. nov. (Chrysophyceae). (manuscript written for submission to *Nordic Journal of Botany*).
- Brett, R.W., Potter, D. and Andersen, R.A. Phylogenetic relationships of the Eustigmatophyceae based upon the 18S rRNA gene, with emphasis on *Nannochloropsis* and with the description of *Nannochloropsis hommersandii* sp. nov. (submitted to *J. Phycol.*)
- Potter, D., Saunders, G.W. and Andersen, R.A. Phylogenetic relationships of the Raphidophyceae and Xanthophyceae as inferred from nucleotide sequences of the 18S ribosomal RNA gene. (manuscript written, for submission to *Amer. J. Bot.*).
- Andersen, R.A., Saunders, G.W., Hill, D.J., Potter, D., and Sexton, J.P. The apparent polyphyly of bioluminescence in dinoflagellates. (in preparation).
- Andersen, R.A. and Potter, D. Phylogenetic relationships of coccolithophorids (Haptophyceae). (in preparation).

Technical Reports

None Applicable

Presentations

Potter, D., LaJeunesse, T.C., Saunders, G.W. and Andersen, R.A. Title: Evolutionary relationships of coccoid marine ultraplankton based upon 18S ribosomal RNA gene sequences: morphological similarity belies broad biological diversity. Phycological Society of America/American Society of Limnology and Oceanography, Miami, FL, June 1994.

(also, probably one presentation at PSA meeting (July 1996), one presentation at ISEP meeting (August 1996).

Invited Presentations

- Andersen, R.A. Title: Chromophytes, Another Lineage of Plants. International Phycological Forum, Tsukuba, Japan. August 25, 1993. [Invited Symposium Speaker]
- Andersen, R.A. Title: Lineages of chromophyte algae previously classified as chrysophytes: what is a chrysophyte? XV International Botanical Congress, Tokyo August 29, 1993. [Invited Symposium Speaker]

- Andersen, R.A. Title: Evidence for a chromophyte lineage with a highly reduced flagellar apparatus. Botanical Institute, University of Copenhagen, Denmark. November 19. 1993.
- Andersen, R.A. Title: Phylogenetic relationships of chromophyte algae based upon cladistic analyses of combined traditional and molecular data sets. Linnean Society, London April 14, 1994. [Invited Symposium Speaker]
- Saunders, G.W., Potter, D. and Andersen, R.A. Title: A molecular approach towards elucidating phylogenetic relationships among the heterokont chromophytes. Fifth International Phycological Congress, Qingdao, China, July 1994. [Invited Symposium Speaker]
- Potter, D., Saunders, G.W. and Andersen, R.A. Title: Phylogenetic relationships of chromophyte algae: evidence from 18S rRNA gene sequences and ultrastructural and biochemical data. Willi Hennig Society, August, 1994. [Invited Symposium Speaker]
- Andersen, R.A. Title: Biodiversity of Photosynthetic Picoplankton. CNRS Productivité Systèmes Océaniques Oligotrophes, Villefranche-sur-Mer, France, January 12, 1995. [Plenary Lecture]
- Andersen, R.A. Title: Phylogenetic relationships of chromophyte algae. Marine Biotechnology Institute, Kamaishi, Japan February 1, 1996. [Invited Symposium Speaker]
- Andersen, R.A. Title: Phylogenetic relationships of chromophyte algae based upon 18S rRNA and *rbcL* gene sequences. University of Tsukuba, Tsukuba Science City, Japan February 5, 1996. [Invited Seminar]
- Andersen, R.A. Title: Algal Biodiversity and Its Significance. Northeast Algal Symposium, Woods Hole, MA. (to be given April 20, 1996). [Distinguished Lecturer]
- Andersen, R.A. Title: Algal biodiversity and the significance of algae in today's world. International Environmetric Society, San Paulo, Brazil (to be given July 1996). [Invited Speaker]
- Andersen, R.A. Title: The role of biodiversity in phycology. 1st European Phycological Congress, Köln, Germany (to be given August 1996). [Plenary Lecture]
- Andersen, R.A. Title: What to do about Protists? Commemorative Conference (150th Anniversary) of the Royal Botanic Gardens, Melbourne, Australia (to be given October 1996). [Plenary Lecture]
- Andersen, R.A., Daugbjerg, N. and Potter, D. Title: Evolutionary relationships of chromophyte algae. Sixth International Phycological Congress, Leiden, Germany (to be given July 1997). [Invited Symposium Speaker]

Book Chapters

None Applicable

Figure 1.
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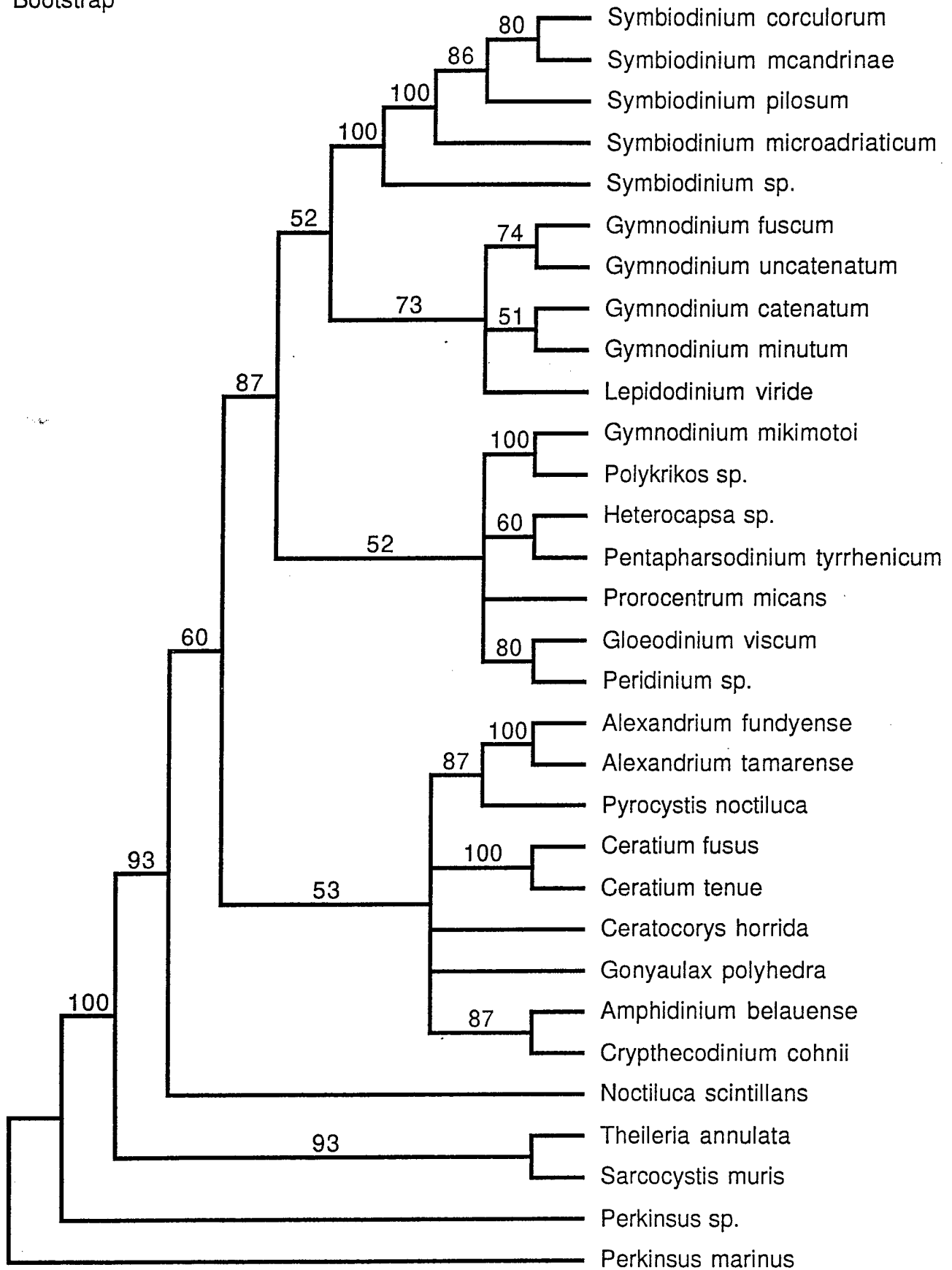
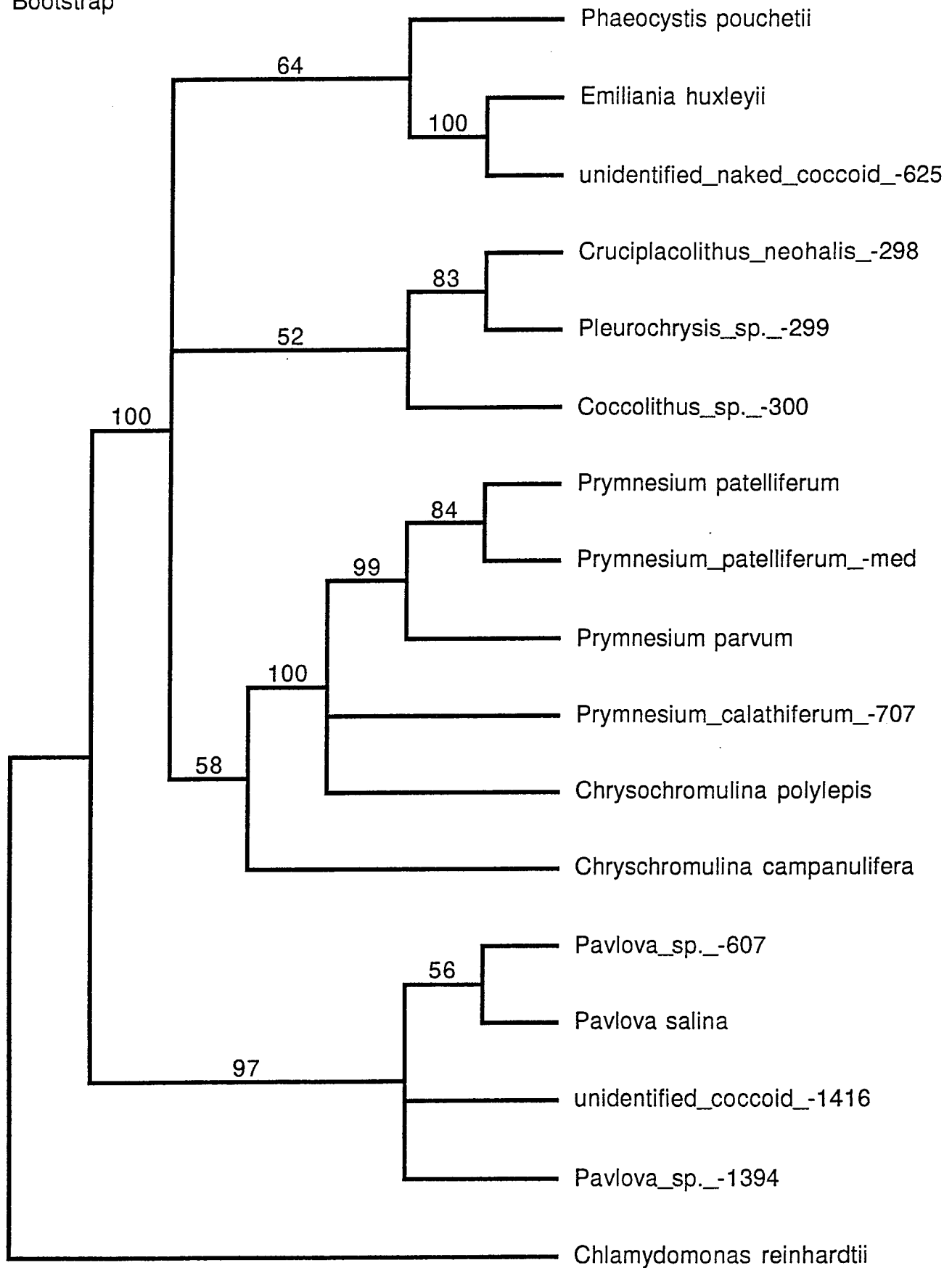


Figure 2.
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13. ABSTRACT (Maximum 200 words) The tasks outlined in the ONR Grant entitled "Marine Phytoplankton Culture Collection: Resources for Algal Biotechnology" have been completed. The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) has provided both starter cultures and large volume batch cultures for ONR scientists working in the areas of algal biotechnology and nanofabrication. In addition, we provided culturing assistance via the telephone and e-mail for ONR scientists. We also trained one ONR-supported Ph.D. graduate student who took our intensive course on Marine Phytoplankton Culturing Techniques. We also isolated into culture new strains of marine phytoplankton that should be useful in the areas of biotechnology and nanofabrication. Finally, the grant provided minor support for the general operations of the CCMP. Our "List of Strains" was just published as a supplement to the Journal of Phycology and was supported in part by this grant.					
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